A *C9orf72* promoter repeat expansion in a Flanders-Belgian cohort with disorders of the frontotemporal lobar degeneration-amyotrophic lateral sclerosis spectrum: a gene identification study

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See Comment page 25

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Summary

Background Amyotrophic lateral sclerosis (ALS) and frontotemporal lobar degeneration (FTLD) are extremes of a clinically, pathologically, and genetically overlapping disease spectrum. A locus on chromosome 9p21 has been associated with both disorders, and we aimed to identify the causal gene within this region.

Methods We studied 305 patients with FTLD, 137 with ALS, and 23 with concomitant FTLD and ALS (FTLD-ALS) and 856 controls from Flanders (Belgium); patients were identified from a hospital-based cohort and were negative for mutations in known FTLD and ALS genes. We also examined the family of one patient with FTLD-ALS previously linked to 9p21 (family DR14). We analysed 130 kbp at 9p21 in association and segregation studies, genomic sequencing, repeat genotyping, and expression studies to identify the causal mutation. We compared genotype-phenotype correlations between mutation carriers and non-carriers.

Findings In the patient-control cohort, the single-nucleotide polymorphism rs28140707 within the 130 kbp region of 9p21 was associated with disease (odds ratio [OR] 2·6, 95% CI 1·5–4·7; p=0·001). A GGGGCC repeat expansion in *C9orf72* completely co-segregated with disease in family DR14. The association of rs28140707 with disease in the patient-control cohort was abolished when we excluded GGGGCC repeat expansion carriers. In patients with familial disease, six (86%) of seven with FTLD-ALS, seven (47%) of 15 with ALS, and 12 (16%) of 75 with FTLD had the repeat expansion. In patients without known familial disease, one (6%) of 16 with FTLD-ALS, six (5%) of 122 with ALS, and nine (4%) of 230 with FTLD had the repeat expansion. Mutation carriers primarily presented with classic ALS (10 of 11 individuals) or behavioural variant FTLD (14 of 15 individuals). Mean age at onset of FTLD was 55·3 years (SD 8·4) in 21 mutation carriers and 63·2 years (9·6) in 284 non-carriers (p=0·001); mean age at onset of ALS was 54·5 years (9·9) in 13 carriers and 60·4 years (11·4) in 124 non-carriers. Postmortem neuropathological analysis of the brains of three mutation carriers with FTLD showed a notably low TDP-43 load. In brain at postmortem, C9orf72 expression was reduced by nearly 50% in two carriers compared with nine controls (p=0·034). In familial patients, 14% of FTLD-ALS, 50% of ALS, and 62% of FTLD was not accounted for by known disease genes.

Interpretation We identified a pathogenic GGGGCC repeat expansion in *C9orf72* on chromosome 9p21, as recently also reported in two other studies. The GGGGCC repeat expansion is highly penetrant, explaining all of the contribution of chromosome 9p21 to FTLD and ALS in the Flanders-Belgian cohort. Decreased expression of *C9orf72* in brain suggests haploinsufficiency as an underlying disease mechanism. Unidentified genes probably also contribute to the FTLD-ALS disease spectrum.

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Introduction

Amyotrophic lateral sclerosis (ALS) and frontotemporal lobar degeneration (FTLD) are two fatal neurodegenerative diseases without effective therapies. ALS is the most common neurodegenerative motor neuron disorder[1] and FTLD has a similar prevalence to Alzheimer's disease in people younger than 65 years.[2] These diseases are two extremes of a spectrum of clinically, pathologically, and genetically overlapping disorders,[3] which suggests an overlap in disease mechanisms. Patients with ALS have reduced control of voluntary muscle movement due to progressive motor neuron degeneration in the motor cortex, brainstem, and spinal cord, resulting in muscle weakness and disturbances of speech, swallowing, or breathing. Up to 50% of patients with ALS have mild disturbances in executive function and some develop overt FTLD.[4,5] Symptoms of FTLD include behavioural, personality, and language disturbances and cognitive

dysfunction caused by degeneration of frontal and temporal cortical neurons. Patients with advanced FTLD can also present with clinical signs of ALS.[6] Although the diseases affect different neurons, TAR DNA-binding protein-43 (TDP-43) is a major constituent of neuronal deposits in both ALS and TDP-43-positive FTLD (FTLD-TDP), the most common pathological FTLD subtype.[7,8]

Family-based linkage and population-based association studies have identified common genetic factors underlying ALS and FTLD. For example, mutations in the ALS genes *TARDBP* and *FUS* are occasionally noted in patients with FTLD[8–10] and mutations in *VCP* (which is associated with an FTLD-related disorder) have been detected in ALS.[11] Most striking is the finding that ALS and FTLD can occur within the same family or even the same patient. In more than 15 families worldwide, autosomal-dominant ALS and FTLD are causally linked with a major disease locus on chromosome 9 (*ALSFTD2* locus).[12–18] The minimal region linked in all these families is about 3·6 Mbp long and contains five known protein-coding genes (*C9orf11, MOBKL2B, IFNK, C9orf72,* and *LINGO2;* figure 1). Moreover, recent genome-wide association studies in ALS cohorts of different European origins have provided evidence for a major genetic risk factor in the same chromosome 9p region.[19–21] We aimed to identify the disease-associated mutation from genetic studies of a family with FTLD-ALS conclusively linked to the 9p21 region and in an extended well characterised patient-control cohort from Flanders (Belgium).[13] After initiation of our study, a Finnish study narrowed the associated region to a

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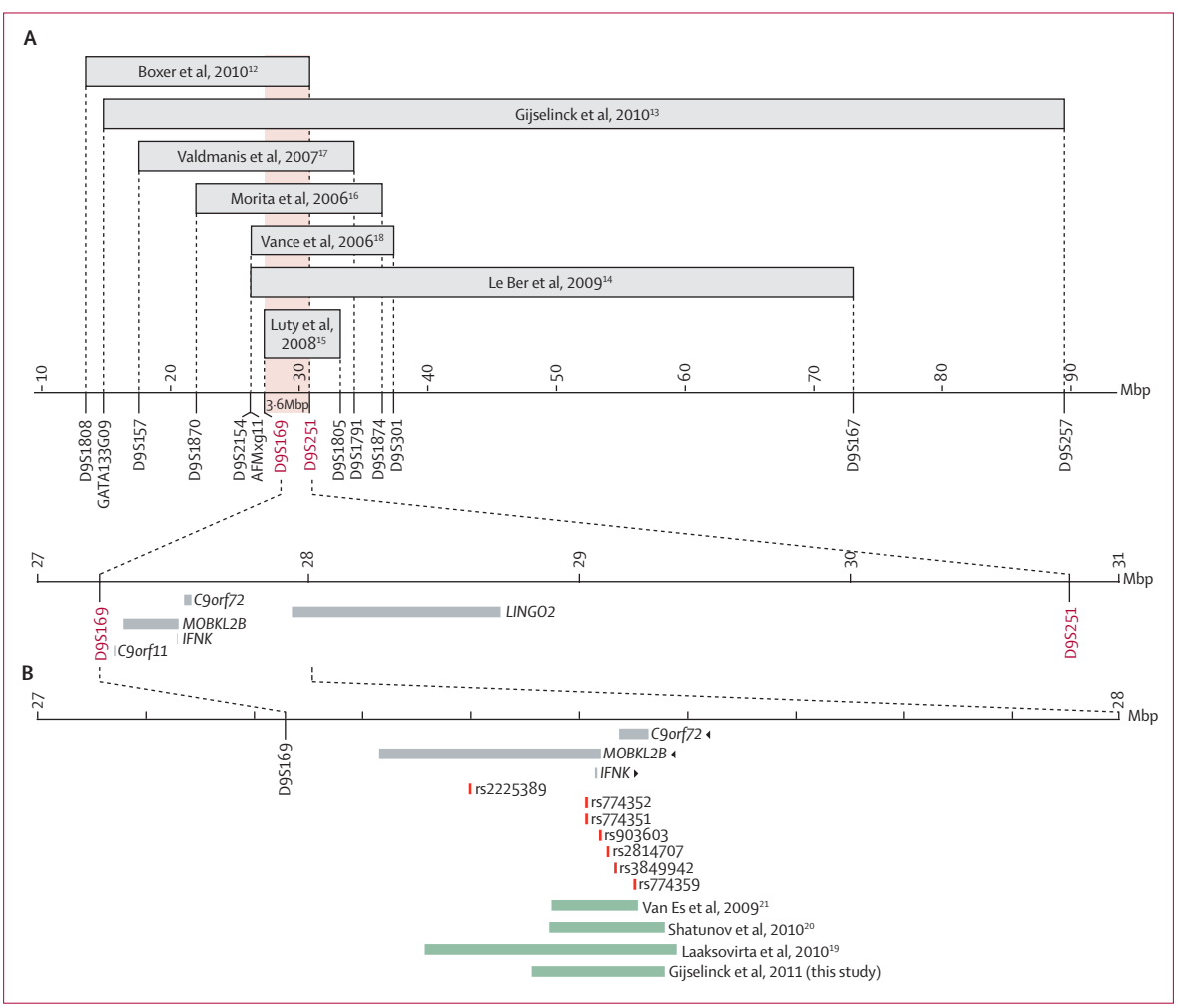

Figure 1: **Schematic representation of the chromosome 9p21 FTLD-ALS locus**
(A) Grey boxes above the genetic map show the minimal candidate regions in all reported FTLD-ALS families conclusively linked to 9p21. The overlap (orange box) defines a minimal interval of 3·6 Mbp between the short tandem repeat markers D9S169 and D9S251, containing five protein-coding genes (*C9orf11*, *MOBKL2B*, *IFNK*, *C9orf72*, and *LINGO2*) shown with grey horizontal lines underneath the minimal candidate region. (B) Single-nucleotide polymorphisms reported to be associated with ALS and FTLD in genome-wide association studies are shown in red and linkage disequilibrium blocks or fine-mapped association regions are shown with green bars. The 130 kbp region analysed in this study is also shown in green; three genes (*MOBKL2B, IFNK*, and *C9orf72*) are located in the associated region. FTLD=frontotemporal lobar degeneration. ALS=amyotrophic lateral sclerosis.

	FTLD (n=337)	FTLD-ALS (n=23)	ALS (n=141)	Controls (n=859)
Age (years)*	62·9 (9·7)	62·6 (10·0)	59·9 (11·6)	65·3 (14·8)
Sex (male)	188 (56%)	10 (43%)	85 (60%)	357 (42%)
Positive family history	101 (30%)	7 (30%)	16 (11%)	..
Autopsy diagnosis	21 (6%)	3 (13%)	5 (4%)	..
FTLD-associated genes				
GRN	24 (7%)	0	NA	0
C9orf72	21 (6%)	7 (30%)	13 (9%)	0
MAPT	4 (1%)	0	NA	NA
VCP	2 (1%)	0	0	NA
CHMP2B	1 (<1%)	0	NA	NA
PSEN1	1 (<1%)	0	NA	NA
ALS-associated genes				
C9orf72	21 (6%)	7 (30%)	13 (9%)	0
SOD1	NA	0	0	NA
TARDBP	NA	0	1 (1%)	NA
FUS	NA	0	1 (1%)	NA
ATXN2	NA	0	2 (1%)	NA
Total mutations	53 (16%)	7 (30%)	17 (12%)	NA

Data are mean (SD) or n (%). ALS=amyotrophic lateral sclerosis. FTLD=frontotemporal lobar degeneration. ..=not applicable. NA=not assessed. *Age at onset or age at inclusion (for controls).

Table 1: **Demographic and genetic characteristics of the Flanders-Belgian cohort**

232 kbp linkage disequilibrium block containing three known genes (*MOBKL2B*, *IFNK*, and *C9orf72*).[19] Another such study implicated the same chromosomal region in an FTLD-TDP cohort,[22] which was confirmed in other FTLD and FTLD-ALS cohorts.[23] Two recent studies reported a GGGGCC repeat expansion in *C9orf72* as the underlying genetic cause in chromosome 9 linked FLTD and ALS.[24,25]

Methods

Participants and study design

The Flanders-Belgian cohort consisted of 337 patients with FTLD, 141 with ALS, and 23 with concomitant FTLD and ALS (FTLD-ALS).[26,27] Patients with FTLD were enrolled from 1998 onwards through an ongoing multicentre collaboration of neurology departments and memory clinics in Belgium (Hospital Network Antwerp Middelheim and Hoge Beuken, University Hospital Antwerp, University Hospitals Leuven, and University Hospital Ghent). Patients with ALS were recruited through the neuromuscular reference centres of the University Hospital Antwerp and the University Hospitals Leuven.[27,28] Additional patients were included who had initially been referred to our Diagnostic Service Facility (Department of Molecular Genetics, VIB, Antwerp, Belgium) for medical genetic testing. Patients were diagnosed by use of a standard protocol and established clinical criteria.[6,26,27,29] Table 1 provides a description of the demographic and genetic characteristics of the cohort and proportions of patients in whom disease diagnosis was confirmed postmortem.

See Online for webappendix

DR14 is an extended FTLD-ALS multiplex family of one of the index patients with FTLD in the Flanders-Belgian cohort; we have previously sampled 29 individuals from this family for genome-wide linkage studies and shown linkage to chromosome 9.[13] Also, we recruited several family members of 10 *C9orf72* mutation carriers of the cohort for genetic studies. In previous analyses of known FTLD and ALS genes, we identified pathogenic mutations in 32 (9%) of 337 patients with FTLD and four (3%) of 141 patients with ALS, but no pathogenic mutations in patients with FTLD-ALS. For the purpose of this study, in which we aimed to identify the chromosome 9 disease gene, we included only those patients from our cohort who did not carry known disease mutations (ie, 305 patients with FTLD, 23 with FTLD-ALS, and 137 with ALS). We included 859 age-matched individuals in the control cohort; these participants were from the same geographical region, did not have a personal or family history of neurodegenerative or psychiatric diseases, and had a mini-mental state examination score of more than 26.

All participants provided written informed consent for participation in clinical and genetic studies. Clinical study protocols and informed consent forms were approved by the local medical ethics committees of the collaborating clinical centres. Genetics study protocol and informed consent forms were approved by the medical ethics committees of the University of Antwerp.

Procedures

For genotyping, we used genomic DNA of two patients and of two control relatives without disease haplotype of family DR14. We selected five candidate genes in the minimal linked candidate region of chromosome 9p21 (figure 1) for mutation analysis by sequencing the whole coding sequence of complementary DNA (cDNA) or sequencing the exons and exon-intron boundaries of genomic DNA. We further defined and sequenced 61 kbp of the most conserved sequences throughout the minimal *ALSFTD2* locus. Whole genome sequencing of four disease haplotype carriers of family DR14 was done with cPAL sequencing technology (Complete Genomics, Mountain view, CA, USA).[30,31] Variations segregating on the disease haplotype were tested in at least 90 (for common variants; ie, minor allele frequency ≥1%) or 856 (for rare variants; ie, minor allele frequency <1%) unrelated controls.

For association analysis, we aimed to replicate the results from ALS and FTLD genome-wide association studies by genotyping the single-nucleotide polymorphism (SNP) rs2814707 (one of the most widely studied significant SNPs; figure 1) in the Flanders-Belgian cohort and in the controls (webappendix p 8). We genotyped short tandem repeat (STR) markers flanking the *C9orf72* GGGGCC repeat in individuals from 10 families with ALS and FTD (10 index patients were part of the Flanders-Belgian cohort, but relatives were

not), segregating the repeat expansion for haplotype sharing, segregation, and multipoint linkage analysis.

We assayed the repeat expansion with a repeat-primed PCR as described elsewhere[32,33] with a gene-specific fluorescently labelled forward primer in *C9orf72* exon 1a, a first reverse primer consisting of four GGGGCC repeats units, and an anchor sequence and a second reverse primer composed of the anchor sequence.

For quantification of *C9orf72* brain transcripts, we used quantitative real-time PCR (qPCR) of amplicons in exons 2–3 to detect both *C9orf72* isoforms a and b, in exons 9–10 to detect isoform a (NM_018325), and in exon 5 (3′-untranslated region) to detect isoform b (NM_145005), all quantified against four housekeeping genes. We used postmortem frontal brain tissue of two index patients with FTLD who carried the *C9orf72* repeat expansion (DR14.1 and DR29.1), two patients with FTLD-TDP who did not have this repeat expansion, and nine controls. We calculated relative expression levels by comparing normalised quantities between patients and controls. We did PCR on cDNA of frontal cortex of DR14.1 and DR29.1 with primers amplifying the complete coding region of the *C9orf72* isoform a transcript. The PCR products were sequenced to establish the transcribed alleles on the basis of the presence of the coding polymorphism rs10122902 in exon 8. Genotypes of cDNA sequences were compared with genomic DNA sequences.

We used western blotting on brain lysates with three different polyclonal antibodies against C9orf72 (made by AbGent, GeneTex, and Santa Cruz [all based in CA, USA]) to compare protein expression in patients DR14.1 and DR29.1 with that in controls.

We did neuropathological analysis of three patients with the *C9orf72* GGGGCC repeat expansion (DR14.1, DR29.1, and DR52.1). One patient (DR25.1) had a *GRN* IVS1+5G>C mutation[34] and one patient (DR40.3) had a *VCP* Arg159His mutation;[35] both patients were negative for the *C9orf72* repeat expansion.

The webappendix contains further technical details of procedures used.

Statistical analysis

We calculated the Hardy-Weinberg equilibrium of the genotyped SNPs as described elsewhere[36] and used a logistic regression model in SPSS version 16.0 to establish association with the genotyped SNPs. We estimated haplotype frequencies in the linkage disequilibrium block with a progressive expectation maximisation insertion algorithm in Haplo stats version 1.2.2. We calculated haplotype associations and sliding window analyses with score statistics in the Haplo stats package. We did multipoint parametric linkage analysis with Allegro version 2.0.[37]

We calculated relative gene expression levels between patients and control individuals with a non-parametric Mann-Whitney U test. For anticipation analysis we did a Breslow (generalised Wilcoxon) test to establish significance and used Student *t* tests to compare mean age at onset between subgroups of patients.

Role of the funding source

The sponsors of the study had no role in study design, data collection, data analysis, data interpretation, or writing of the report. The corresponding author had full access to all the data in the study and had final responsibility for the decision to submit for publication.

Results

We excluded mutations in all five protein-coding genes in the 3·6 Mbp long minimal region (figure 1) through exon-based sequencing in family DR14. Subsequently, we sequenced 61 kbp of the most conserved non-coding sequences in the minimal region without finding patient-specific variants segregating with disease in family DR14. Whole-genome sequencing identified 120 new sequence variations in the minimal candidate region that segregated with disease, 37 of which were absent in controls.

We investigated genetic associations with chromosome 9p21 by use of SNP rs2814707 in the Flanders-Belgian cohort of patients with FTLD, FTLD-ALS, and ALS (table 1) and matched controls. We noted a significant association of this SNP with disease in the overall cohort (odds ratio [OR] 2·6, 95% CI 1·5–4·7; p=0·001), which was mainly derived from the combined subgroup of patients with ALS and FTLD-ALS (3·5, 1·7–7·3; p=0·001; webappendix p 8).

To fine-map the 9p21 association signal we constructed a high-density SNP map of a 130 kbp linkage disequilibrium block containing rs2814707 defined in the European CEU population (residents of UT, USA, with northern and western European ancestry; figure 1). 492 variants were identified by sequencing of the complete 130 kbp region in a selection of 15 patients with ALS and FTLD-ALS carrying the risk allele of rs2814707 in a homozygous or heterozygous state. We genotyped 58 SNPs capturing all this genetic variation (webappendix pp 9–10). Linkage disequilibrium mapping and haplotype association analysis showed significant association in linkage disequilibrium block 1 containing three genes—*C9orf72*, *IFNK*, and *MOBKL2B* (webappendix p 8)—that was most significant in the ALS and FTLD-ALS subgroup (p=0·004; webappendix pp 11–12). We used sliding window analysis in the subgroup of patients with ALS and FTLD-ALS to fine-map the associated region further to a locus comprising only the gene *C9orf72* (p<0·0001; webappendix p 14). In family DR14, whole-genome sequencing identified only one variation in the 130 kbp region, which was an intronic 1 bp insertion located deep in the first intron of *MOBKL2B* (IVS1+27228_+27229insT) with no obvious functional effect.

Inspection of sequence reads of *C9orf72* suggested that only one allele of exon 1a, the first non-coding exon of the

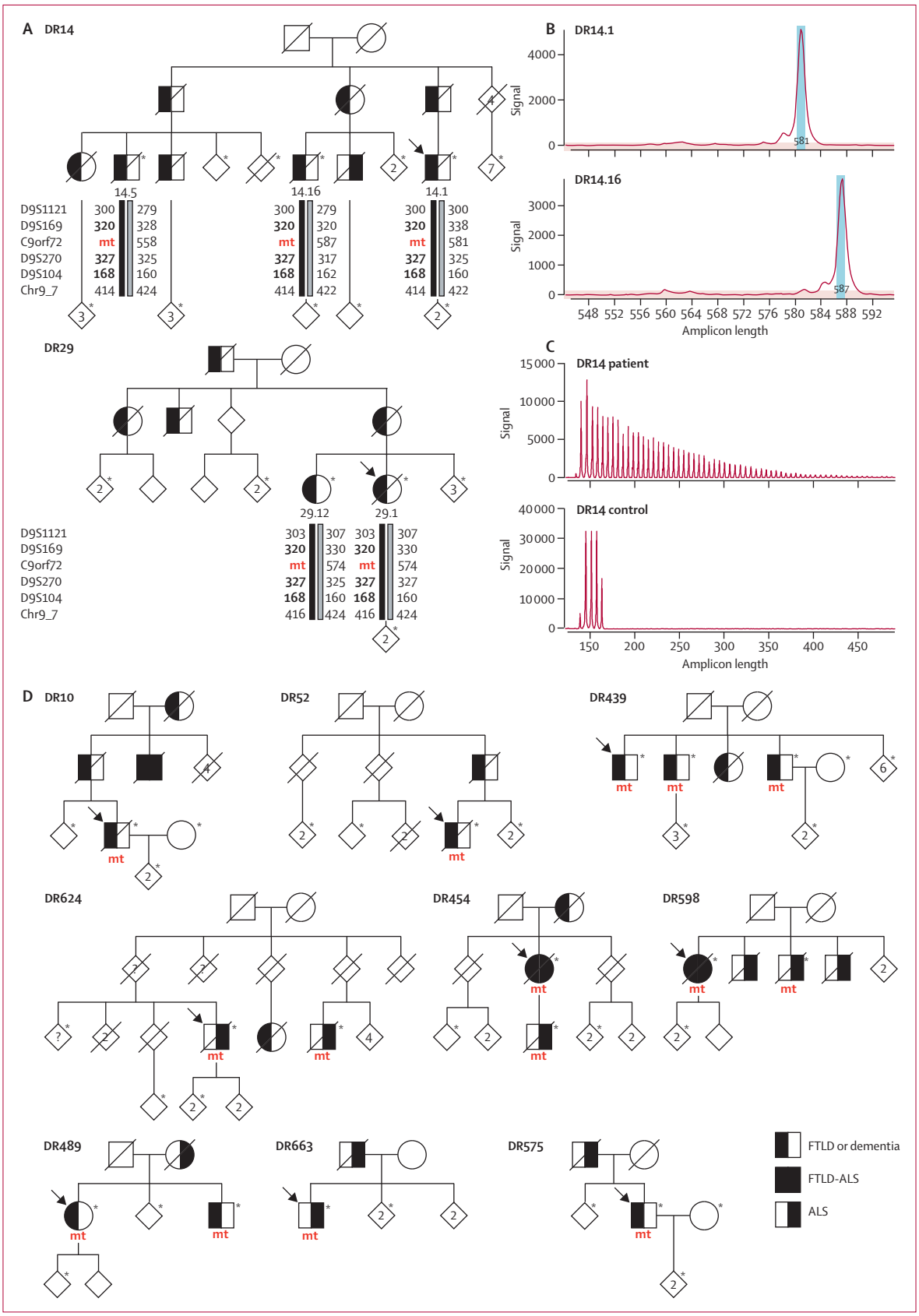

***Figure 2: C9orf72* repeat expansion in FTLD, ALS, and FTLD-ALS families**

(A) Abbreviated pedigrees of genetically related families DR14 and DR29. Disease haplotypes segregating in the families are shown as black bars and the haplotype shared between families DR14 and DR29 are shown in bold. The *C9orf72* repeat is included in the haplotype with mt representing the repeat expansion on the mutant allele. Male (squares), female (circles), and deceased (diagonal black lines) individuals are shown. Individuals with obtainable DNA are shown with an asterisk to the right of the symbol. Numbers in diamonds show the number of unaffected at-risk children. The proband of the family is indicated with an arrow. Sex, birth order, and mutation status of unaffected individuals have been omitted for reasons of confidentiality. (B) Chromatograms of the *C9orf72* repeat in two patients of family DR14 showing absence of segregation resulting from hemizygosity of the normal allele owing to loss of the mutant allele with the *C9orf72* repeat expansion. Blue bars show allele size (with corresponding number in parentheses). (C) Results from one patient and one control in family DR14 with the repeat-primed PCR amplification assay showing the expansion of the GGGGCC repeat as multiple peaks, each representing a different sized amplification product. (D) Pedigrees of small families segregating the *C9orf72* repeat expansion. FTLD=frontotemporal lobar degeneration. ALS=amyotrophic lateral sclerosis.

longest *C9orf72* transcript (isoform a), was sequenced in patients in the DR14 family compared with non-affected control relatives (data not shown). This finding became clearer from the non-segregation pattern obtained with the polymorphic GGGGCC hexanucleotide repeat that is located just upstream of exon 1a and was contained in the exon 1a PCR amplicon (figure 2). Because we initially expected a deletion, we used an exon-based, quantitative dosage analysis (webappendix p 15). The dosage quotient of exon 1a showed high variability ranging between 0 and 1 in patients and control relatives in family DR14, suggestive of a genomically unstable region with variable PCR amplification efficiency. Subsequently, we used a repeat-primed amplification assay, and identified a large expansion of the GGGGCC repeat that segregated with disease in family DR14 (figure 2). This expansion explained the conflicting segregation data we had obtained in the GGGGCC repeat analysis by a loss of the expanded allele in the PCR amplification of exon 1a.

Screening for the GGGGCC repeat expansion in patients without mutations in known FTLD or ALS genes resulted in mutation frequencies of 7% in FTLD (21 of 305 individuals), 30% in FTLD-ALS (seven of 23 individuals), and 9% in ALS (13 of 137 individuals; table 2). In patients, the repeat expansion exceeded the upper detection limit of the assay we used, suggesting a minimum length of about 60 repeats, whereas in 856 controls the maximum repeat length was 25 units. Exclusion of patients carrying the GGGGCC repeat expansion from the overall patient cohort abolished any significant association with rs2814707 in the overall analysis and in the different subgroups of patients (webappendix p 8).

	Patients	*C9orf72* repeat expansion
FTLD		
Positive familial history	75	12 (16%)
Isolated	230	9 (4%)
Total	305	21 (7%)
FTLD-ALS		
Positive familial history	7	6 (86%)
Isolated	16	1 (6%)
Total	23	7 (30%)
ALS		
Positive familial history	15	7 (47%)
Isolated	122	6 (5%)
Total	137	13 (9%)

Positive familial history was defined as at least one first-degree relative with disease of the FTLD-ALS spectrum. Isolated was defined as apparently sporadic patients (ie, patients with no affected first-degree relative) or patients for whom no information on family history was available. Patients in whom other ALS or FTLD genes were identified were excluded from this analysis. ALS=amyotrophic lateral sclerosis. FTLD=frontotemporal lobar degeneration.

Table 2: **Frequency of *C9orf72* repeat expansion carriers in the Flanders-Belgian cohort**

	Sex	Family history	Age at onset (years)	Age (years)	Disease duration (years)	Diagnosis	FTLD phenotype	ALS phenotype	Initial ALS symptoms
DR14.1	Male	Familial	57	60 (at death)	3	FTLD	bvFTD	..	..
DR29.1	Female	Familial	51	55 (at death)	4	FTLD	bvFTD	..	..
DR52.1	Male	Familial	53	58 (at death)	5	FTLD	bvFTD	..	..
DR55.1	Female	Sporadic	42	48 (at death)	6	FTLD	bvFTD	..	..
DR439.1	Male	Familial	69	72 (current age)	>3	FTLD	bvFTD	..	..
DR489.1	Female	Familial	45	48 (current age)	>3	FTLD	bvFTD	..	..
DR659.1	Male	Familial	46	48 (current age)	>2	FTLD	bvFTD	..	..
DR661.1	Male	Familial	53	54 (current age)	>1	FTLD	bvFTD	..	..
DR670.1	Male	Sporadic	58	66 (at death)	8	FTLD	bvFTD	..	..
DR671.1	Male	Sporadic	49	66 (at death)	17	FTLD	bvFTD	..	..
DR393.1	Female	Familial	65	69 (at death)	4	FTLD-ALS	bvFTD	UMN/LMN	Bulbar
DR396.1	Female	Familial	60	62 (at death)	2	FTLD-ALS	bvFTD	UMN/LMN	Bulbar
DR454.1	Female	Familial	69	72 (at death)	3	FTLD-ALS	bvFTD	UMN/LMN	Spinal
DR598.1	Female	Familial	65	68 (at death)	3	FTLD-ALS	PNFA	UMN/LMN	Bulbar
DR681.1	Male	Familial	50	53 (at death)	3	FTLD-ALS	bvFTD	LMN	Bulbar
DR504.1	Male	Familial	60	61 (at death)	1	ALS	..	UMN/LMN	Bulbar
DR519.1	Female	Familial	47	53 (current age)	>6	ALS	..	UMN/LMN	Spinal
DR624.1	Male	Familial	55	65 (at death)	10	ALS	..	UMN/LMN	Spinal
DR664.1	Female	Sporadic	62	63 (at death)	1	ALS	..	UMN/LMN	Spinal
DR667.1	Male	Sporadic	38	39 (at death)	1	ALS	..	UMN/LMN	Spinal
DR669.1	Male	Unknown	65	66 (current age)	>1	ALS	..	UMN/LMN	Spinal

FTLD=frontotemporal lobar degeneration. ALS=amyotrophic lateral sclerosis. bvFTD=behavioral variant of FTLD. UMN/LMN=upper and lower motor neuron signs. LMN=predominantly lower motor neuron signs. PNFA=progressive non-fluent aphasia.

Table 3: **Clinical characteristics of carriers of the *C9orf72* repeat mutation**

We have obtained data for relatives of ten index patients with a *C9orf72* repeat expansion including patients with FTLD, FTLD-ALS, and ALS. In these ten families, we showed segregation of the GGGGCC repeat expansion with disease (figure 2). Haplotype sharing analysis with polymorphic STR markers showed that one family (DR29) shared a maximal region of 3·5 Mbp with family DR14, suggesting that both families descended from a common ancestor (figure 2). All six documented patients of family DR29 had presented with FTLD without ALS. Linkage analysis in five informative families (DR14, DR29, DR454, DR439, and DR598) resulted in a maximal summed multipoint logarithm of the odds (LOD) score of 5·78 at marker D9S1833. In these families, taking into account censoring for unaffected mutation carriers, the median onset age of 21 patients from the third generation was 58 years (95% CI 55·8–60·2), which was younger than was that of nine patients from the second generation (69 years, 63·2–74·8; p=0·074; webappendix p 17).

Most mutation carriers in the Flanders-Belgian cohort had a positive family history of disease (table 2). The remaining mutation carriers consisted of apparently sporadic patients (three with FTLD and five with ALS) and patients for whom no information on family history could be obtained (six with FTLD, one with FTLD-ALS, and one with ALS). None of the repeat expansion carriers had a mutation in any of the other known FTLD or ALS genes (table 1), including the recently reported *UBQLN2*.[38] The mean onset age of disease in the 41 patients with pathogenic GGGGCC repeat expansions was 55·5 years (SD 8·9, range 38–71), and was in the same range for 21 patients with FTLD (55·3 years; SD 8·4, range 42–71), seven patients with FTLD-ALS (57·9 years; 9·7, 43–69), and 13 patients with ALS (54·5 years, 9·9, 38–64). In patients with FTLD, the onset age in mutation carriers was significantly earlier than it was in 284 patients without the expansion mutation (63·2 years; 9·6, 29–83; p=0·001). In the ALS cohort, 13 mutation carriers also had an earlier mean onset age compared with 124 non-carriers (60·4 years; 11·4, 32–79), although the difference was not significant (p=0·075).

Detailed clinical records were available for 21 carriers of the *C9orf72* mutation (10 had FTLD, five had FTLD-ALS, and six had ALS; table 3). We made a diagnosis of the behavioural variant of FTLD (bvFTD) in 14 of 15 patients with FTLD and FTLD-ALS. The ALS phenotype in *C9orf72* mutation carriers was consistent with that of classic ALS (10 of 11 patients), consisting of

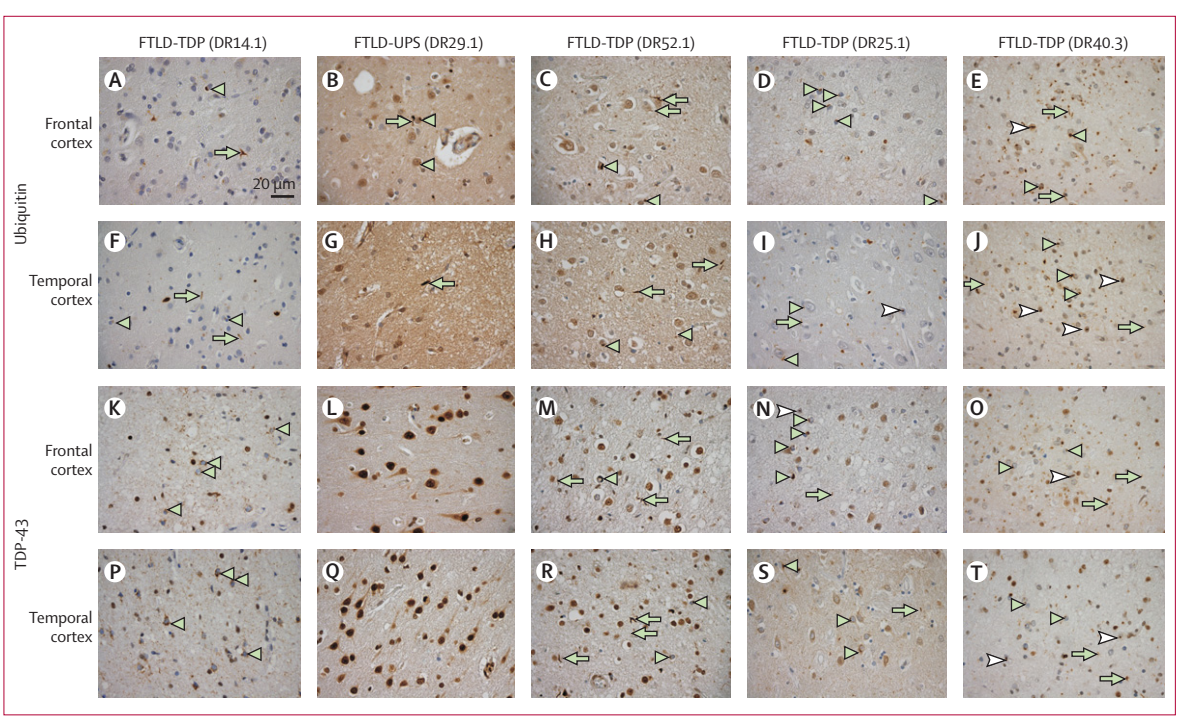

Figure 3: **TDP-43 and ubiquitin immunohistochemistry in carriers of the GGGGCC repeat expansion with FTLD**
Ubiquitin (A–J) and TDP-43 (K–T) immunohistochemistry of frontal and temporal cortices from three patients (DR14.1, DR29.1, and DR52.1) with FTLD and the GGGGCC repeat expansion and patients with FTLD-TDP with a *GRN* mutation (DR25.1) or a *VCP* mutation (DR40.3). Ubiquitin-positive dystrophic neurites (arrow) and NCI (arrowhead) are present in the frontal (A–C) and temporal (F–H) cortices of patients DR14.1, DR29.1, and DR52.1. TDP-43 immunostaining of samples from patients DR14.1 (K and P) and DR52.1 (M and R) showed both NCI and dystrophic neurites in temporal cortex and frontal cortex consistent with FTLD-TDP type B pathology. Ubiquitin lesions were more frequently identified than were TDP-43 lesions in these two patients. Ubiquitin staining of tissue from patient DR29.1 showed NCI and dystrophic neurites (B and G), whereas TDP-43 immunoreactive lesions were absent (L and Q), consistent with a diagnosis of FTLD-UPS. By comparison with the GGGGCC repeat expansion carriers, the ubiquitin (D, E, I, and J) and TDP-43 pathology (N, O, S, and T) in DR25.1 and DR40.3 was more extensive. Ubiquitin and TDP-43 immunoreactive neuronal intranuclear inclusions (white arrowhead) were noted only in DR25.1 and DR40.3 and were absent in carriers of the GGGGCC repeat expansion. FTLD=frontotemporal lobar degeneration. TDP-43=TAR DNA-binding protein-43. NCI=neuronal cytoplasmic inclusions. UPS=ubiquitin proteasome system.

signs of upper and lower motor neuron disease, apart from one patient with FTLD-ALS who developed a predominantly lower motor neuron disorder. The onset site of ALS was spinal in six patients and bulbar in five patients.

Neuropathological findings were assessed according to the most recent TDP-43 classification. In FTLD probands DR14.1 and DR52.1, the relatively low lesion load and the presence of neuronal intracytoplasmic inclusions, and to a lesser extent dystrophic neurites, in all layers of frontal and prefrontal temporal cortices is compatible with type B TDP-43 proteinopathy (figure 3).[13,39] The TDP-43 lesion load in DR14.1 and DR52.1 was less overt and extensive than that in patients with *GRN* or *VCP* mutations (figure 3). In proband DR29.1, who was diagnosed with FTLD-UPS (ie, FTLD with inclusions detectable by use of immunohistochemistry for proteins in the ubiquitin proteasome system and negative for TDP-43), ubiquitin immunohistochemistry showed immunoreactive neuronal cytoplasmic inclusions, dystrophic neurites, and, to a lesser extent, neuronal intranuclear inclusions in the hippocampus and frontal cortex (figure 3). TDP-43 labelling in this patient showed only a few neuronal intracytoplasmic inclusions in the dentate gyrus.

The GGGGCC repeat is located 5′ adjacent to *C9orf72* exon 1a in the promoter, as shown by the presence of histone marks, DNase-I hypersensitive clusters, and transcription factor binding sites (ENCODE data;[40] webappendix p 16). DNA sequences directly flanking and possibly including the GGGGCC repeat can bind several factors: subunits of the RNA polymerase II complex, which mediates transcription of protein-coding genes (eg, *RPB1*); components of basal transcription factor II D (TFIID); that is, a scaffolding complex that binds to the core promoter of the TATA box in class II promoters, to allow assembly of the transcription machinery (eg, TATA-binding protein and TBP-associated factor 1); and many regulatory transcription factors. Therefore the GGGGCC repeat is most likely to be located in the core promoter of *C9orf72*.

We analysed the effect of the GGGGCC repeat expansion on *C9orf72* expression in frontal cortex samples from probands of families DR14 and DR29 and compared the results with that expression in nine controls (figure 4) and two patients with FTLD-TDP without repeat expansion. qPCR with a fragment located in both isoforms (a and b) and a specific fragment in isoform a or isoform b (figure 4) showed a 50% reduced expression of *C9orf72* specifically in both repeat expansion carriers (p=0·034). Loss of allele-specific transcript expression was shown by use of a silent coding SNP in exon 8 (rs10122902) of which the C allele segregates on

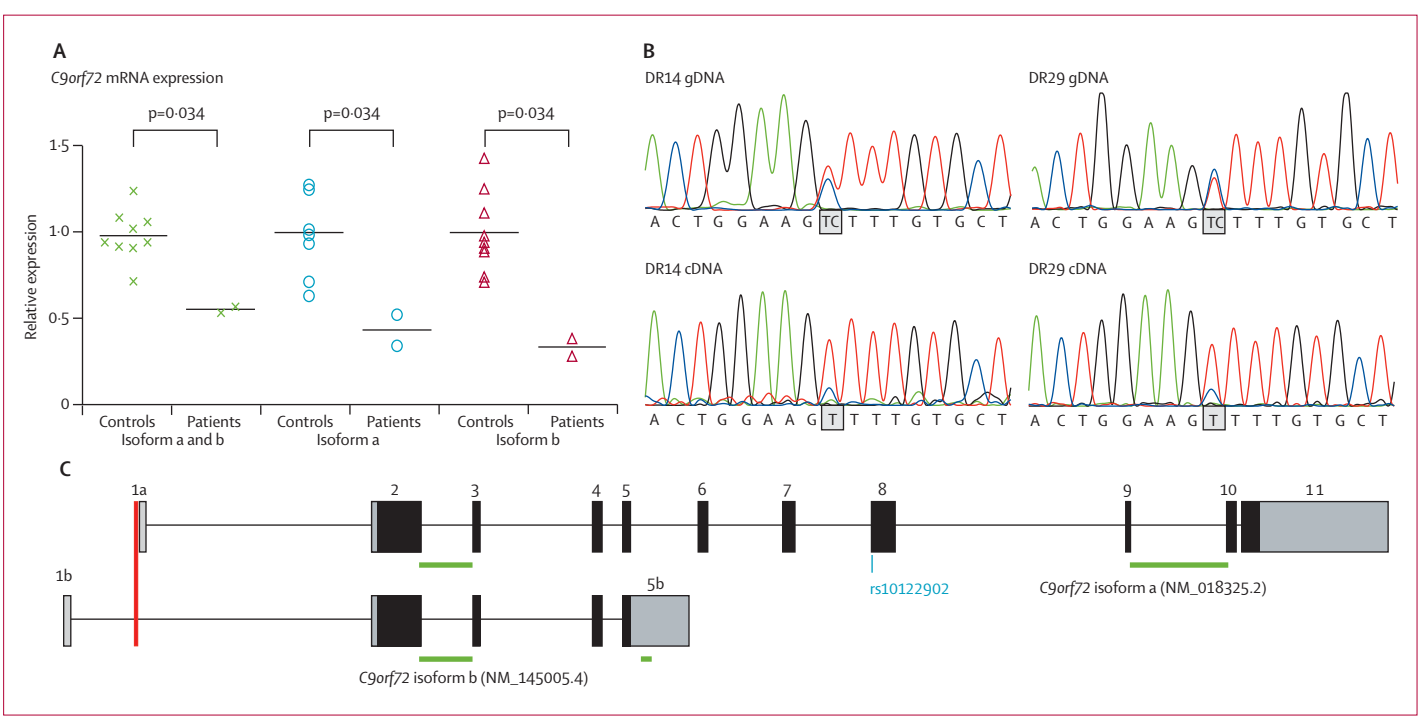

***Figure 4: C9orf72* transcript expression analyses**

(A) Quantitative real-time PCR analysis of mRNA of frontal cortex normalised against four housekeeping genes to compare *C9orf72* expression in DR14 and DR29 probands with that in nine control individuals. We used three different assays, which detected either both isoforms a and b, or each isoform separately. Relative expression levels are shown for every individual brain sample. The horizontal line shows the mean level. p values were calculated with a non-parametric test. (B) Sequence traces obtained for rs10122902 in exon 8 are shown for genomic DNA (gDNA) and frontal cortex complementary DNA (cDNA) of probands DR14.1 and DR29.1. (C) Schematic presentation of the two major transcripts of *C9orf72*. Coding regions are shown in black and non-coding regions are shown in grey. The position of the expanded GGGGCC repeat is shown with a red vertical bar. Green horizontal bars show the location of the PCR amplicons used in the quantitative real-time PCR analysis. rs10122902 in exon 8 (shown in blue) was used to track *C9orf72* normal and mutant transcripts by sequencing.

***Panel:* Research in context**

Systematic review

We searched PubMed for articles published in any language between Jan 1, 1995, and Oct 1, 2011, with the search terms "chromosome 9" AND "FTD", "chromosome 9" AND "ALS", "GWAS" AND "ALS", "repeat expansion", and "repeat expansion detection PCR". We also searched in the reference lists of included papers. We incorporated reports of families with concomitant frontotemporal lobar degeneration (FTLD) and amyotrophic lateral sclerosis (ALS) that was conclusively linked to chromosome 9 to reduce the linked candidate region, and published ALS and FTLD-TDP genome-wide association studies and replication studies to define the smallest region associated in the same locus. The repeat-primed PCR method for screening the expansion was applied as described elsewhere for fragile X.[32]

Interpretation

FTLD-ALS has been described in several families with genetic linkage to a locus at chromosome 9. Several population-based association studies in ALS have pointed to the same chromosomal region . While we were writing this paper, two other groups published reports[24,25] suggesting that a pathogenic hexanucleotide repeat expansion in the gene *C9orf72* underlies the published linkages and associations in both ALS and FTLD. The repeat mutation was the most common genetic cause of FTLD and ALS in these studies. We present the independent finding of a hexanucleotide repeat expansion in an extended FTLD-ALS family conclusively linked with chromosome 9p21. We showed that the mutation frequency is as high as 86% in familial patients with FTLD-ALS, 47% in those with ALS, and 16% in those with FTLD, which are similar to those in the two other reports.[24,25] Furthermore, we replicated the published genome-wide association with the chromosome 9 locus in our Flanders-Belgian cohort, in which we also fine-mapped the associated region to the *C9orf72* gene only. We correlated the repeat expansion with specific neuropathological characteristics of reduced or even absent TDP-43 neuropathology. Further studies are needed to identify the mechanism by which the mutation leads to disease. Finally, 62% of patients with familial FTLD cannot have their disorder explained by a mutation in *C9orf72* or another gene, whereas this value is 50% in ALS. This finding suggests that other genetic causes exist for both diseases.

the disease haplotype. Both the DR14 and DR29 probands were heterozygous C/T on genomic DNA but near homozygous for the T allele on cDNA (figure 4), indicating specific loss of transcription of the mutant allele carrying the expanded GGGGCC repeat and suggesting a *cis*-acting effect of the GGGGCC repeat expansion on *C9orf72* expression. Expression analyses in lymphoblast cell lines of several mutation carriers and controls were inconclusive because of highly variable *C9orf72* expression levels (data not shown).

In lysates from frontal and temporal cortex of the DR14 and DR29 probands, immunoblotting with three different polyclonal anti-C9orf72 antibodies did not show appreciable differences in protein expression compared with four controls, but high background signals suggested that non-specific binding was making the data unreliable (data not shown). Immunohistochemical staining showed increased cytoplasmic granular immunoreactivity and globular neuronal cytoplasmic inclusions (webappendix pp 19), which were absent in a control individual and reduced in brain tissue from a patient with FTLD-TDP and a *GRN* mutation.[34] Immunocytochemistry of endogenous C9orf72 in SH-SY5Y neuroblastoma cells showed predominant cytoplasmic immunoreactivity with weak nuclear staining (webappendix p 20). Overexpression of either enhanced green fluorescent protein-tagged or untagged C9orf72 (isoforms a and b) showed diffuse nuclear and cytoplasmic reactivity (webappendix p 20).

Discussion

Previous genetic linkage and association studies have established a major locus for the spectrum of ALS and FTLD disorders on chromosome 9p21. In two reports,[24,25] a pathological GGGGCC repeat expansion in *C9orf72* was shown to be the underlying genetic defect. Here, we report the independent finding of the same GGGGCC repeat expansion in patients with FTLD, FTLD-ALS, and ALS in a Flanders-Belgian cohort (panel). We identified several lines of evidence for the causative relation between the repeat expansion and disease. First, we showed complete segregation of the expanded repeat with disease in a family conclusively linked with FTLD-ALS (family DR14)[13] and in ten smaller families with FTLD or FTLD-ALS. One of these families, DR29, shared a 3·5 Mbp disease haplotype with family DR14, supporting a common ancestor. Second, the GGGGCC repeat expansion in *C9orf72* in family DR14 was the only mutation in the 130 kbp region of chromosome 9p21 segregating with disease and was absent in controls, apart from one deep intronic 1 bp insertion in intron 1 of *MOBKL2B* with no obvious functional relevance. Third, in the Flanders-Belgian cohort, we noted a highly significant genetic association in a small region containing *C9orf72* as the only gene in ALS and FTLD-ALS subgroups. Comparison of the risk haplotype with data from genome-wide association and replication studies in ALS[19–21,23,41] and FTLD-TDP[22] confirmed that the same haplotype was underlying disease association in the Flanders-Belgian cohort. Fourth, the genetic association with chromosome 9p21 was wholly explained by the GGGGCC repeat expansion. Extensive mutation analysis of the associated 130 kbp region in 15 unrelated patients carrying the chromosome 9p21 risk haplotype identified no other variants that could explain the association.

The GGGGCC repeat expansion was identified in 41 (9%) of 465 patients without mutations in known FTLD and ALS genes in the Flanders-Belgian cohort. The frequencies were highest in familial FTLD-ALS (86%) and ALS (47%), in which the repeat expansion was the most common genetic cause. In the total FTLD subgroup, an expanded GGGGCC repeat was the second most common cause of disease after *GRN* mutations.[34] Although few patients with ALS and FTLD were analysed compared with the number of patients with FTLD, mutation frequencies in our cohort are very similar to those reported elsewhere.[24,25] In our study, the GGGGCC repeat expansion in *C9orf72* was the only mutation identified in the group of patients with FTLD-ALS. Together, these findings suggest that *C9orf72* has a key role in the FTLD-ALS spectrum.

Symptoms of FTLD in carriers of the mutation were mainly behavioural, which is equivalent to other reports.[24] The motor neuron disease phenotype in mutation carriers was mostly consistent with classic ALS. We cannot exclude that some patients with FTLD had subtle subclinical signs of ALS and might develop the disease at later stages. Conversely, thorough neuropsychological testing of patients with ALS might show mild executive dysfunction or mild behavioural abnormalities. Notably, in patients with FTLD, four mutation carriers had a positive family history of ALS, and one ALS mutation carrier had relatives with dementia. Further detailed genotype-phenotype studies will clarify the range of phenotypes associated with *C9orf72* mutations. Why some of the repeat expansion carriers develop ALS whereas others develop FTLD is unknown. Presently unknown genetic factors might render specific neuronal populations more susceptible to a pathological effect. In our cohort, patients carrying an expanded repeat showed highly variable ages of disease onset and disease duration. Age of FTLD onset was younger in patients carrying a repeat expansion than it was in non-carriers. The wide variability in onset age in mutation carriers is suggestive of modifying factors. In this context, the previously identified locus on chromosome 14 showing borderline significant linkage in a genome-wide study of family DR14[13] might harbour a disease-modifying factor.

Pathological analysis of patients who have FTLD with a *C9orf72* repeat expansion established a diagnosis of FTLD-TDP type B in two individuals and of FTLD-UPS in one individual. Compared with patients who had FTLD and a *GRN* or *VCP* mutation, the TDP-43 lesion load was reduced in patients with FLTD and the *C9orf72* mutation. The finding of FTLD-TDP type B pathological features is in agreement with previous findings.[12,24,42] In *C9orf72* brains there was more ubiquitin-positive staining than there was TDP-43 staining (ie, TDP-43 load was reduced), suggesting the presence of a yet unknown inclusion protein, as previously suggested.[12,24,42] Notably, in patients in our cohort with pathologically confirmed FTLD-TDP, the repeat expansion explained two (15%) of 13 familial cases, which is a smaller proportion than was reported in other cohorts.[24]

We estimated that the expanded GGGGCC repeat contained at least 60 repeat units, corresponding to the detection limit of the repeat-primed assay we used. The sizes of the repeat expansion in patients did not overlap with those observed in 856 controls, in whom the repeat contained fewer than 25 units. The absence of large repeat expansions in controls suggests that the disease mutation is fully penetrant. In our study, the mutation frequency was about five times higher in familial patients than it was in isolated patients. However, mutation frequencies were also comparatively high in isolated patients with FTLD-ALS (6%), ALS (5%), and FTLD (4%). In addition to limited documentation of family history in complex adult onset diseases, this difference in frequencies might in part be explained by a comparatively high de novo expansion rate of the GGGGCC repeat. However, because few relatives of apparently isolated patients were available for analyses, we could not examine this rate. Moreover, because of the very low mutation frequency in the general population, carriers of the homozygous repeat expansion are expected to be rare and consequently we cannot conclude whether homozygosity of the repeat expansion is associated with a more severe phenotype or lethality.

Because the exact sizes of expanded alleles were not established, we could not assess the correlation of repeat size with severity of disease expressed as onset age or age at death. However, we reported a trend towards younger age of onset between generations. This trend could point to the presence of anticipation, but owing to sample size constraints we could not fully take into account familial correlations, and factors such as recall bias or improved diagnostics might have confounded the finding.

The expanded repeat is located in the core promoter region of *C9orf72*, which binds the RNA polymerase II complex and regulatory transcription factors. Whether the GGGGCC repeat sequence is directly involved in binding of the basal transcription complexes, recruitment of regulatory transcription factors, or other regulatory functions is unknown; however, its location predicts an effect on transcription regulation. Accordingly, expression of mRNA of the two major *C9orf72* transcripts in the frontal cortex of two carriers of the FTLD expansion was reduced to about 50% compared with expression in the frontal cortex of control individuals and two patients with FTLD but not the GGGGCC repeat expansion. Allele-specific expression analysis further showed a *cis*-acting effect of the expanded repeat on the expression of the mutant allele. Our expression data are similar to previous findings;[24] however, that study did not include an expression analysis of the shorter isoform b. The location of the expanded GGGGCC repeat and the significantly reduced *C9orf72* expression in brain suggest that haploinsufficiency owing to aberrant promoter function or chromatin structure, resulting in about 50% reduced transcript, is the pathological mechanism. Western blot analysis of brain lysates of repeat expansion carriers with three different polyclonal C9orf72 antibodies failed to provide reliable data for the effect of reduced transcript expression on protein expression, most likely because of the low specificity of available antibodies. We did not find evidence for the presence of other loss-of-function mutations in *C9orf72* through exon-based sequencing.

Other disease mechanisms described for non-coding repeat expansions in neurodegenerative diseases[43,44] cannot formally be excluded: for example, aberrant splicing of *C9orf72* primary transcripts and sequestration of RNA-binding proteins by the expanded repeat could disturb their normal function in processing of the RNA of other genes. RNA toxicity by accumulation of repeat

expansion containing transcripts in RNA foci might disturb cell function, as preliminarily reported elsewhere[24] in the nucleus of frontal cortex and spinal cord of carriers of the *C9orf72* expansion. However, these mechanisms can apply only to the shorter major transcript b, as the repeat is absent from the isoform a transcript.

Although the gene is highly conserved in all vertebrates, the function of *C9orf72* is unknown. On the basis of evidence from co-expression studies, researchers suggest that it might be involved in ubiquitin-dependent protein degradation, which is known to be involved in proteinopathies including those of TDP-43 (webappendix p 18). Our preliminary data for endogenous *C9orf72* expression showed predominant cytoplasmic immunoreactivity with weak nuclear reactivity, corresponding with another report,[24] and our overexpression experiments showed diffuse expression of C9orf72 protein in both the cytoplasm and nucleus. This finding might partly explain reports of immunoreactivity in the nucleus.[25] This discrepancy in locations of immunoreactivity might be the result of the use of commercially available antibodies that were not characterised or tested for specificity.

In conclusion, we showed that the chromosome 9 linkage is explained by a GGGGCC repeat expansion in the promoter of *C9orf72,* a gene with unknown function. This repeat mutation is the most common cause of ALS with and without FTLD in the Flanders-Belgian population, and is the second most common cause of pure FTLD after *GRN* mutations. Nevertheless, the genetic cause of FTLD and ALS remains unexplained in many patients. Taken together with mutation screening data in other genes linked with FTLD-TDP (eg, *GRN* and *VCP*), 23% of familial FTLD-TDP is unexplained, suggesting the presence of other genetic factors that are yet to be identified. The repeat expansion decreases the expression of *C9orf72* in brain and its effect on protein expression remains to be clarified; more specific antibodies are needed to characterise the localisation and quantity of the C9orf72 protein. The identification of this gene provides firm evidence that FTLD and ALS share common disease mechanisms and might lead to better insights into FTLD and ALS disease mechanisms and to disease-modifying treatments.

Contributors

IG, JvdZ, GK, MC, and CVB designed the study. IG, TVL, JvdZ, KS, GK, JJ, SPe, AS, MC, and CVB participated in writing of the report. IG, TVL, MC, and CVB did the literature search. IG, TVL, JvdZ, KS, SPh, GK, JJ, KB, CVC, SPe, MC, and CVB worked on the figures. TVL, JvdZ, SE, PDJ, RV, PS, JDB, MM, KP, WR, PC, and PPDD obtained samples from patients or clinical data. All authors participated in collection, analysis, or interpretation of the data.

Conflicts of interest

We declare that we have no conflicts of interest.

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